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Production of Chimeric Mice by Exchanging Nuclei from Blastomeres of the Two-Cell Embryo

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ABSTRACT—We report a new method for generating chimeras without removal of the zona pellucida. Chimeric mice were produced by exchanging nuclei from blastomeres of two-cell embryos between BALB/c and C3Hf mice. Of 108 experimental embryos, 70 (65%) survived successful exchange of nuclei between blastomeres of the two-cell embryos. Of the 70 operated embryos, 48 (69%) developed to blastocysts. Moreover, from the 154 operated two-cell embryos that were transferred to oviducts, 19 mice developed of which three were chimeras. The three chimeric mice contained both BALB/c and C3Hf mouse cells and the two types of cell were almost equally distributed. These results demonstrate that chimeras can be obtained by exchanging nuclei from blastomeres of two-cell embryos between BALB/c and C3Hf mice.

INTRODUCTION

Chimeric embryos have been invaluable in studies of developmental mechanisms [15], sex determination [6], cell-cell interactions [10], and the rescue of otherwise lethal phenotypes [11]. In general, chimeras are produced by two methods, one involving aggregation of embryos, as described by Tarkowski [12], and the other involving microsurgical injection of blastocyst-stage embryos, as described by Gardner [4]. For production of aggregation chimeras, the zona pellucida is removed from embryos, which then aggregate. After development to morula or blastocysts, the embryos are transferred to an intact uterus while they are still free of the zona pellucida. The zona pellucida of mouse embryos plays an important role in their successful transfer to and development in the oviduct [1, 7].

McGrath and Solter [5] reported a new procedure for pronuclear transplantation in the mouse. Their procedure includes the removal of pronuclei with an enucleation pipette without penetration of the plasma membrane. The fusion of the isolated nuclei, which are surrounded by cytoplasm and plasma membrane, with a previously enucleated egg is then induced by Sendai virus (HVJ). This method allows the exchange of nuclei among blastomeres. The nucleus of one blastomere in the two-cell embryo can be exchanged for another nucleus from a blastomere at the same stage. In this way, chimeras can be produced by nuclear transplantation. In this report, we describe a new method by which nuclear transplantation is used to generate chimeras without removal of the zona pellucida.

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MATERIALS AND METHODS

Mice

C3Hf (GPI-1B) mice were gift of Dr. K. Tanaka, Osaka University. ICR and BALB/c (GPI-1A) mice were obtained from SLC, Inc. (Shizuoka, Japan). Most mice were used at 8 to 10 weeks of age.

Micromanipulation of eggs

Female mice were induced to superovulate by intraperitoneal injections of 7.5 i.u. of pregnant mare's serum, which were followed 48 hr later by an injection of 7.5 i.u. of human chorionic gonadotrophin. Female mice were paired overnight with male mice and vaginal plugs were checked the following morning. Two-cell embryos were flushed from the anterior portion of the uterine horns 24 hr later with Hepes-buffered Whitten's medium [16].

The experimental approach, presented schematically in Fig. 1, was as follows. Microsurgery was performed with micromanipulators (MO-102N; Narishige, Japan) and a fixed-stage microscope (IMT-2; Olympus, Japan). Holding and enucleation pipettes were fashioned from capillary tubing (outer diameter, 1.0 mm; inner diameter, 0.65 mm) by the method of McGrath and Solter [5].

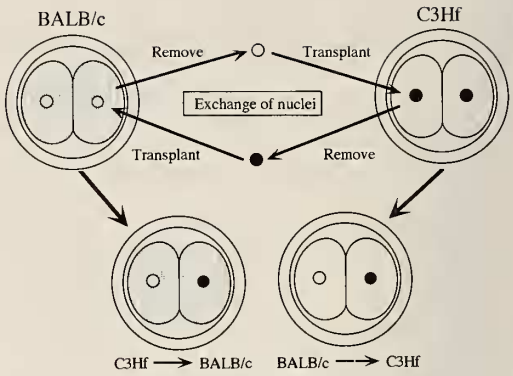

FIG. 1. Formation of chimeric eggs by nuclear transplantation

Before microsurgery, embryos were incubated for 15 to 45 minutes at 37°C in Hepes-buffered Whitten's medium that contained cytochalasin B (5 μg/ml) and colcemid (0.1 μg/ml). Six to eight drops of this medium and one drop of a suspension of

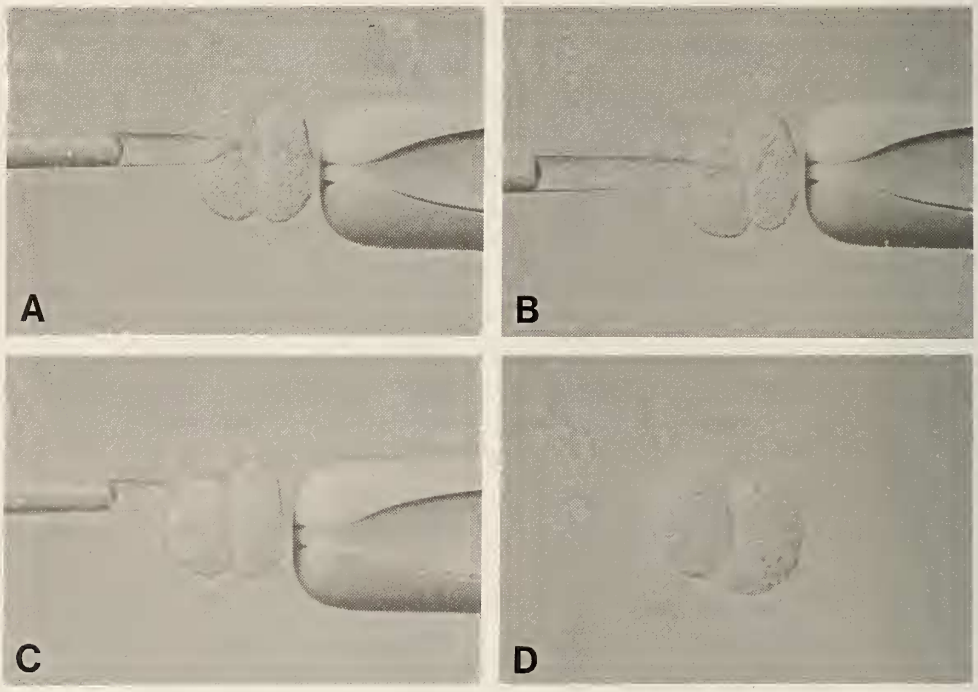

FIG. 2. Micromanipulation of eggs.
A, The two-cell embryo was secured by a holding pipette and the zona pellucida was penetrated with an enucleation pipette. B, The nucleus was aspirated into the enucleation pipette together with a small portion of the plasma membrane. C, The nucleus and HVJ were injected into the perivitelline space of a two-cell embryo from which one nucleus had been removed in advance. D, The nucleus fused with the two-cell embryo during subsequent incubation.

HVJ (3000 hemagglutinating units per milliliter) that had been inactivated by treatment with β-propiolactone were covered with paraffin oil. Embryos were then placed singly in six to eight hanging drops. Each two-cell embryo was secured by a holding pipette and the zona pellucida was penetrated with an enucleation pipette (Fig. 2A). Penetration of the plasma membrane was avoided and the pipette was advanced into one blastomere of the 2-cell embryo, at a point adjacent to the nucleus. A small volume of cytoplasm, containing the entire nucleus, was drawn into the pipette (Fig. 2B). The enucleation pipette was moved to the second drop which contained HVJ, and a volume was aspirated that was approximately equal to that of the nuclear karyoplast. The enucleation pipette was then moved to a third drop that contained the previously enucleated blastomere and intact blastomere of different two-cell embryo, and was inserted into the perivitelline space through the site of the previous enucleation. The nuclear karyoplast and HVJ were injected and the enucleation pipette was withdrawn (Fig. 2C). The embryo was washed several times with Whitten's medium and incubated in the same medium at 37°C for a few hours (Fig. 2D). The nuclear karyoplast fused with the enucleated blastomere during incubation. The fused embryos were then used for experiments.

Culture of eggs

Two-cell embryos were cultured for four days in 50-μl drops of modified Whitten's medium under paraffin oil at 37°C in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2, and the percentage of blastocysts was determined. One blastocyst was transferred to 10 μl of Dulbecco's modified Eagle's medium (DMEM) that contained 10% fetal calf serum (FCS) in a capillary tube and cultured at 37°C in an atmosphere of 5% CO_2 and 95% air for 4 days for outgrowth of the trophoblast. After outgrowth of the trophoblast, the embryo was placed in 5 μl of lysis buffer (50 mM Tris, 1 mM EDTA ,1 mM 2-mercaptoethanol, pH 7.5) and lysed by three cycles of freezing and thawing. The lysate was used as the source of material for analysis of isozymes of glucose phosphate isomerase (GPI-1).

Transfer of embryos

After micromanipulation, the eggs were transferred into the oviducts of day one pseudopregnant ICR mice. Four to seven embryos were transferred into each oviduct. The recipients were allowed to give birth naturally.

Analysis of glucose phosphate isomerase (GPI-1) isozymes

GPI-1 isozymes were analyzed by a modified version of the method of Eppig *et al.* [2]. Pieces of tissue were homogenized with ground-glass microhomogenizers in 50 μl of lysis buffer. Each homogenate was centrifuged for 20 min at 16000× g and the supernatant was used as the source of enzyme. The isozymes were separated on a Titan III Zipzone cellulose acetate plate (Helena Laboratories, Texas) in 0.043 M Tris, 0.046 M glycine buffer (pH 8.5) for 2 hr at 180 V. GPI-1 was detected with a cellulose acetate plate overlay that contained 50 mM Tris-HCl (pH 8.0), 2.5 mM $MgCl_2$, 2.5 mM fructose-6-phosphate, 1 mM NADP, 0.1 mg/ml phenazine methosulphate, 0.5 mg/ml nitroblue tetrazolium and 5 IU/ml glucose-6-phosphate dehydrogenase. After several hours of incubation at 37°C, the staining appeared to be stable. The relative levels of GPI-1A and GPI-1B in each sample were estimated by comparison with reference plates on which different proportions of GPI-1A and GPI-1B had been analysed.

RESULTS

To determine the feasibility of producing chimeras by nuclear transplantation, experiments were conducted by the method of McGrath and Solter [5]. Of 108 experimental embryos, 81 (75%) under went successful enucleation of one blastomere of the two-cell embryo, and 74 (91%) of the resulting nuclear karyoplasts were successfully injected, together with inactivated HVJ, into the perivitelline space of two-cell embryos with single enucleated blastomeres. After incubation at 37°C, the karyoplast had fused to the plasma membrane of the enucleated blastomere in 70 (95%) embryos. The overall efficiency of nuclear transplantation was, therefore, 65%.

TABLE 1. Development of chimeric eggs after nuclear transplantation *in vitro*

Type of egg	No. of cultured embryos	Developmental stage (%)		
		Arrested	Morula	Blastocyst
Operated				
BALB/c → C3Hf	23	1	3	19 (83%)
C3Hf → BALB/c	19	8	2	9 (47%)
ICR → ICR	28	7	1	20 (71%)
Total	70	16	6	48 (69%)
Unoperated				
BALB/c	12	1	1	10 (83%)
C3Hf	11	0	1	10 (91%)
ICR	33	0	1	32 (97%)
Total	56	1	3	52 (93%)

Eggs were cultured for 4 days at 37°C in 5% CO_2, 5% O_2 and 90% N_2.
BALB/c → C3Hf denotes that a nuclear karyoplast of a two-celled embryo from a BALB/c mouse was fused with an enucleated blastomere of a C3Hf mouse embryo at the same stage.
C3HF → BALB/c denotes the reverse.

After microsurgery, experimental and control embryos were cultured for 4 days and the number of embryos that successfully developed to the blastocyst stage was determined (Table 1). Of 56 unoperated embryos, 3 (5%) and 52 (93%) developed to the morula stage and the blastocyst stage, respectively. By contrast, of the 70 operated embryos, 6 (9%) and 48 (69%) developed to the morula stage and the blastocyst stage, respectively.

Figure 3 shows the electrophoretic profile of GPI from embryos with trophoblast outgrowths. BALB/c embryo gave only the AA homodimeric band, and C3Hf embryo gave only the BB homodimeric band. The F_1 (BALB/c×C3Hf) embryos expressed the AA, hybrid AB and BB allozymes. By contrast, the nuclear-transferred embryos gave the AA and BB homodimeric bands but not the AB band.

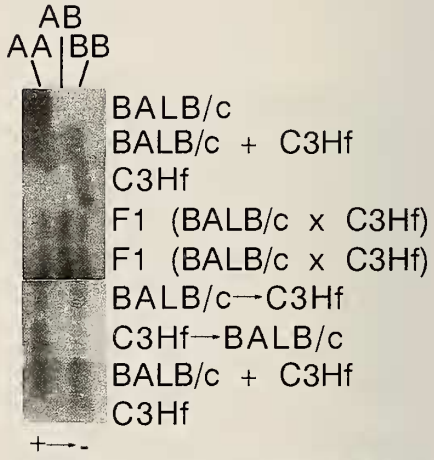

FIG. 3. Electrophoretic profiles of glucose phosphate isomerase from chimeric embryos.
Each lane shows the GPI-1 isozymes of an embryo. Each blastocyst was cultured for 4 days at 37°C in DMEM that contained 10% FCS. After trophoblast outgrowth, each embryo was lysed by freezing and thawing and the lysate was used as the source of GPI-1.

Table 2 summarizes the development of control and operated eggs after transfer to oviducts. Transfer of 173 control two-cell embryos to the oviducts of pseudopregnant females resulted in the implantation of 93 (54%) and in the birth of 46 progeny (27%). By contrast, transfer of 154 operated embryos resulted in the implantation of 63 (41%) and in the birth of 19 progeny (12%). Out of 19 mice, 3 were completely covered with coat of the BALB/c type (albino), 13 were completely covered with coat of the C3Hf type (agouti), and 3 exhibited chimeric coat color. Of the three chimeras, two were phenotypic males and one was female.

Figure 4 shows isozyme patterns for the chimeric mice. The chimeric mice contained both BALB/c and C3Hf mouse cells and the two types of cell

TABLE 2. Postimplantation development of chimeric eggs after nuclear transplantation

Type of egg	No. of transferred embryos	No. of implantation	Pups	
			Phenotype	No.
Operated				
BALB/c → C3Hf	78	33	BALB/c	2
			C3Hf	7
			Chimera	1
C3Hf → BALB/c	76	30	BALB/c	1
			C3Hf	6
			Chimera	2
Total	154	63		19
Unoperated				
BALB/c	103	53	BALB/c	20
C3Hf	70	40	C3Hf	26
Total	173	93		46

Eggs were transferred into the oviduct of day 1 pseudopregnant recipient mice.
BALB/c → C3Hf and C3Hf → BALB/c are explained in the footnote to Table 1.

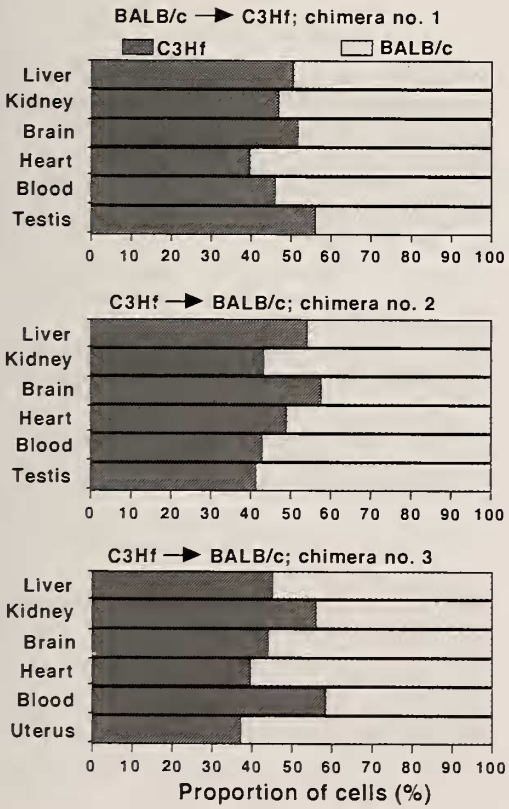

FIG. 4. Distribution of cells in chimeric mice.
The proportion of each type of cell was determined from the relative levels of GPI-1 isozymes. The GPI-1 isozyme of BALB/c is the AA type and that of C3Hf is the BB type.

were distributed almost equally. The three BALB/c-type mice, namely, those with albino hairs, gave only the AA homodimeric band of GPI-1 and the 13 C3Hf-type mice, namely, those with agouti hairs, gave only the BB homodimeric band of GPI-1 when various organs, such as the liver, kidney, brain and blood, were analyzed (data not shown).

DISCUSSION

The results of this study confirm that chimeric embryos can be produced by replacing the nucleus of one blastomere in a two-cell embryo with another nucleus from a blastomere at the same stage. The morphology of the operated embryos, which continued to develop, was essentially the same as that of unoperated normal embryos. The percentage of blastocysts that developed *in vitro* from operated embryos was slightly reduced as compared with that from unoperated embryos. The early development of mouse embryos is reg-

ulated by an intrinsic cytoplasmic program [14]. The physiological relationship between the donor nucleus and recipient cytoplasm may not be totally appropriate. The rate of development to blastocysts *in vitro* was lower in C3Hf → BALB/c, than in BALB/c → C3Hf embryos. However, the implantation rate, the number of young produced and the efficiency of formation of chimeras were similar for C3Hf → BALB/c and BALB/c → C3Hf embryos. Among the operated embryos, the number of progeny was significantly lower than that among control embryos, although implantation rates were not statistically different. This difference is probably due to the reduced rate of development into blastocysts of operated embryos and/or the reduced rate of development after implantation. The reason for the reduction in the rate of development after implantation has not yet been determined. In the production of aggregation chimeras between BALB/cA-nu/nu and C3H/HeN mice, Ohsawa *et al.* [9] reported that 446 aggregated embryos were transferred to recipient mice and 52 offspring were born. Of these offspring, 39 grew to adult mice and 17 were chimeras. Therefore, the efficiency of formation of chimeras was about the same as in the present experiments. However, further experimentation is necessary to explain fully the efficiency of formation of chimeras by this method.

Compared with other methods such as the generation of aggregation chimeras, our procedure has two advantages. First, it can produce two chimeras from two two-cell embryos. In the case of the method involving aggregation of embryos, only one chimera can be generated from two two-cell embryos. The second advantage of our method is that it is not necessary to remove the zona pellucida. Aggregation chimeras are produced after removal of the zona pellucida, which has an important role in the successful transfer to and development of mouse embryos in the oviduct [1, 7]. In sheep, Moor and Cragle [8] found that eight-cell embryos, from which the zona pellucida had been removed enzymatically, failed to continue to develop either *in vitro* or after transfer to recipient ewes. The sheep embryos prior to the late morula stage fail to survive *in vivo* after the zona pellucida had been removed [13]. There is no satisfactory method reported for culture of sheep embryos *in vitro* at early cleavage stages. Production of sheep chimeras by aggregation of blastomeres is more complex and laborious than the method used for the production of murine aggregation chimeras. Usually, sheep embryos are embedded in agar after aggregation for production of chimeras in order to seal any lesions in the zona pellucida, and then they are transferred to the oviduct [3]. Therefore, our method, which does not require removal of the zona pellucida will probably prove more effective and, in addition, more versatile than methods that rely on the simple aggregation of denuded embryos.

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REFERENCES

1 Bronson R, McLaren A (1970) Transfer to the mouse oviduct of eggs with and without the zona pellucida. J Reprod Fertil 22: 129–137

2 Eppig JJ, Kozak LP, Eicher EM, Stevens LC (1977) Ovarian teratomas in mice are derived from oocytes that have completed the first meiotic division. Nature 269: 517–518

3 Fehhilly CB, Willadsen SM, Tucker EM (1984) Experimental chimaerism in sheep. J Reprod Fert 70: 347–351

4 Gardner RL (1968) Mouse chimaeras obtained by the injection of cells into the blastocyst. Nature 220: 596–597

5 McGrath J, Solter D (1983) Nuclear transplantation in the mouse embryo by microsurgery and cell fusion. Science 220: 1300–1302

6 McLaren A (1975) Sex chimaerism and germ cell distribution in a series of chimaeric mice. J Embryol Exp Morphol 33: 205–216

7 Modlinski JA (1970) The role of the zona pellucida in the development of mouse eggs *in vivo*. J Embryol Exp Morphol 23: 539–547

8 Moor RM, Cragle RG (1971) The sheep egg: enzymatic removal of the zona pellucida and culture of eggs in vitro. J Reprod Fertil 27: 401–409

9 Ohsawa N, Kojima H, Yokoyama M (1984) Phenotypes of chimeras between Balb/c A-nu/nu and

C3H/HeN. Exp Cell Biol 52: 111–113

10 Papaiannou VE, Artzt K, Dooher GB, Benner D (1979) Effects of chimerism for two T/t complex mutations on sperm function. Gamete Res 2: 147–151

11 Stevens CL (1978) Totipotent cells of parthenogenetic origin in a chimaeric mouse. Nature 276: 266–267

12 Tarkowski AK (1961) Mouse chimaeras developed from fused eggs. Nature 190: 857–860

13 Trounson AO, Moore NW (1974) The survival and development of sheep eggs following complete or partial removal of the zona pellucida. J Reprod Fert 41: 97–105

14 Waksmundzka M, Krysiak E, Karasiewicz J (1984) Autonomous cortical activity in mouse eggs controlled by a cytoplasmic clock. J Embryol Exp Morphol 79: 77–96

15 West JD (1984) Cell markers. In "Chimeras in developmental biology" Eds by Le Douarin N, McLared A, Academic Press, London, pp 39–67

16 Whitten WK (1971) Embryo medium. Nutrient requirements for the culture of preimplantation embryos *in vitro*. Adv Biosci 6: 129–141